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Immobilised transketolase for carbon–carbon bond synthesis: biocatalyst stability

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Dedicated to: The authors would like to dedicate this paper to the memory of Professor Malcolm Lilly who sadly died in May 1998

Abstract

Transketolase from *Escherichia coli* used for the asymmetric synthesis of carbon–carbon bonds has been immobilised on two commercial supports (Eupergit-C and Amberlite XAD-7). Stabilisation against oxidative affects could only be achieved by the addition of reducing agents. However, immobilisation did have a significant effect on stabilising the enzyme activity against the denaturing effects of the aldehyde acceptor required for the reaction. Using the transketolase catalysed synthesis of L-erythrulose from glycolaldehyde and β-hydroxypyruvate an improvement of 80 and 100 fold was found in the half-life of Amberlite XAD-7 and Eupergit-C immobilised preparations, respectively. Such an improvement may avoid the need for reactant feeding strategies in subsequent reactor designs.

Keywords: Carbon–carbon synthesis; Immobilised transketolase; Biocatalyst stability

1. Introduction

Biocatalysis has received increasing attention in recent years as a powerful tool available to the synthetic chemist. While the use of biological catalysts has become widespread in the laboratory, large scale implementation requires the development of process techniques and a framework within which they can be applied [10,17]. The use of enzyme immobilisation, by attaching the enzyme to a solid support, is one of a number of techniques now established to improve process productivity. Benefits result not only from allowing the enzyme to be retained within the bioreactor, reclaimed from the product stream and/or reused, but also increased operational stability.

Many enzymes lose catalytic activity in the operational environment of a biocatalytic process. This can take the form of denaturation due to the high ionic environment or due to the necessarily high concentrations of one or more reaction components. As a measure of the improvement in enzyme stability achievable via immobilisation, many studies have now shown reduced thermal denaturation of attached enzymes [9]. This increase in stability is often

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attributed to the rigidification of the protein's tertiary structure [4,5]. Gabel [4] also demonstrated an increase in the stability of an immobilised protein in the presence of high concentrations of denaturing chemicals such as urea. In this work we report on the immobilisation of transketolase for use as a synthetic catalyst for carbon–carbon bond synthesis.

Transketolase (EC 2.2.1.1) is a homodimeric, thiamine pyrophosphate dependent enzyme with residues from both monomers bridging its two active sites [11]. The enzyme catalyses the stereospecific transfer of a two carbon ketol group to an aldehyde acceptor [2]. Unlike rabbit muscle aldolase its reactants do not need to be phosphorylated (thereby overcoming the necessity of subsequent dephosphorylation). This results in facile asymmetric carbon–carbon bond formation making the enzyme of particular interest as a catalyst for the production of synthetic sugar molecules.

In recent years the gene sequence for *Escherichia coli* transketolase has been self-cloned into *E. coli* JM107 [3]. The enzyme can be produced in significant quantities by fed-batch fermentation [8] and the three dimensional structure has been resolved to 1.9 Å [12]. Transketolase has also been successfully immobilised, with a reasonable residual activity, onto Eupergit-C®, a commercially available immobilisation support [1]. The free enzyme has also been characterised with respect to reactor selection and operation [14]. One of the major constraints upon the operation of the reactor using soluble transketolase as the biocatalyst is the instability of the enzyme in the presence of the aldehyde acceptor. Both the holo and the apo forms of transketolase were found to be readily denatured by glycolaldehyde even in the presence of reducing agents such as mercaptoethanol. Hence the need to operate a fed-batch reactor in order to minimise the concentration of denaturant in the bioreactor. Immobilisation of transketolase confers a number of process advantages. In this paper we discuss the possibility of overcoming the need for reactant feeding by the immobilisation of transketolase onto commercially available support materials. The model reaction used in this study was the transfer of a two carbon ketol group from β-hydroxypyruvic acid (HPA) (1) to glycolaldehyde (GA) (2) to yield L-erythrulose (ERY) (3) and carbon dioxide (4) (Fig. 1). Although the general reaction scheme for transketolase is reversible, the use of HPA (1) as the ketol donor results in the formation of byproduct carbon dioxide which shifts the equilibrium such that the reaction becomes irreversible. The aldehyde acceptor (GA) has frequently been shown to be the most reactive in substrate specificity studies [2,7].

2. Materials and methods

2.1. Analytical

Biotransformation reactants and products were assayed by HPLC consisting of an ISS-100 autosampler with a Series 200-LC pump (Perkin-Elmer, Beaconsfield, Bucks, UK). Detection was carried out by refractive index using a Shodex RI-71 detector (Showa Denko K.K.,

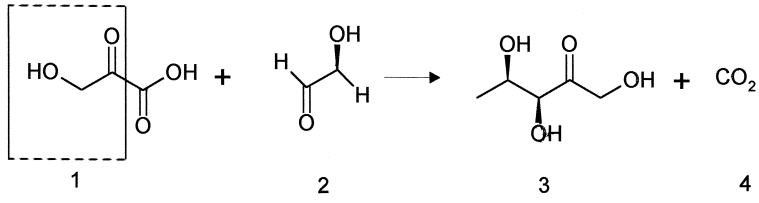

Fig. 1. Reaction scheme for transketolase catalysed carbon–carbon bond synthesis.

Japan). Data collection and integration were performed on a PC driven Prime version 2.2.3 chromatography data station (HPLC Technology, Macclesfield, Cheshire, UK). Conditions for the quantitative analysis of β-hydroxypyruvic acid, glycolaldehyde and L-erythrulose were as reported previously by Mitra and Woodley [13]. Separation was achieved on two Aminex HPX-87H columns in series (300 × 7.8 mm) (Biorad, Hemel Hempstead, Herts, UK). The column temperature was maintained at 65°C in a Model 7970 Block Heater (Jones Chromatography, Hengoed, Mid Glamorgan, UK). Separation was achieved at a flow rate of 0.45 ml min^{-1} in 6 mM H_2SO_4 as the mobile phase.

Spectrophotometric assays were carried out on either a Kontron Uvikon 922 spectrophotometer (Kontron Instruments, Watford, Herts, UK) or a Unicam UV2 spectrophotometer (Unicam, Cambridge, UK). In order to quantify the amount of transketolase activity a variation of the linked enzyme spectrophotometric assay developed by Villafranca and Axelrod [16] was used as reported by French and Ward [3] and Mitra and Woodley [13]. The assay links the activities of α-glycerophosphate dehydrogenase, triosephosphate isomerase, phosphoribose isomerase and D-ribulose-5-phosphate-3-epimerase with transketolase. The quantity of transketolase present was measured by the decrease in reduced nicotinamide adenine dinucleotide (NADH) concentration. The components of the assay were added in the following amounts to the final assay volume of 1.5 ml: 100 mM glycyl glycine buffer (pH 7.6) containing 0.5 mg ml^{-1} bovine serum albumin (BSA), thiamine pyrophosphate (TPP) [2.5 mM], magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) [9 mM], reduced nicotinamide adenine dinucleotide (NADH) [0.145 mM], 0.2 U ml^{-1} α-glycerophosphate dehydrogenase–triosephosphate isomerase, 0.2 U ml^{-1} phosphoribose isomerase and 0.2 U ml^{-1} D-ribulose-5-phosphate-3-epimerase, 50 ml of a suitably diluted sample of transketolase and ribose-5-phosphate (added last) [3 mM]. After the addition of the 50 μl sample containing suitably diluted enzyme the transketolase was allowed to complex by equilibration for 10 min. The reaction was subsequently initiated by the addition of the ribose-5-phosphate. The oxidation of NADH was monitored spectrophotometrically at 340 nm and correlated with the amount of transketolase [6]. Assay conditions were 35°C and pH 7.6. All components of the assay were supplied by Sigma–Aldrich (Poole, Dorset, UK).

Protein assays were done using Biorad protein assay reagent diluted 1:5 with reverse osmosis water. The diluted dye was then filtered to 0.45 μm to remove particulate material. A 30 ml sample containing protein was added to 1.5 ml of the filtered Biorad reagent. This mixture was allowed to stand for at least 10 min after which the absorbance at 595 nm was measured. Values were quantified against BSA standards.

2.2. Transketolase preparation

Transketolase was expressed at high levels in *E. coli* JM107/pQR700 [3] and cells grown as reported previously [8]. Centrifuged cells from fermentation were resuspended at 12.5% w/v in 1 M phosphate buffer at pH values ranging from 6 to 9. The cell extracts were prepared by homogenising the resuspended cells via four passes at 1200 bar in a Lab 40 Homogeniser. Extracts were then clarified by centrifugation at 13,000 rpm for 5 min to yield clarified enzyme solutions containing 250 U ml^{-1} transketolase and 20 g l^{-1} protein.

2.3. Transketolase immobilisation

2.3.1. Eupergit-C

Clarified cell extracts were prepared in 1 M phosphate buffer at pH 6.5 for the holo-enzyme and pH 7.5 for the apo-form at a suitable transketolase concentration (between 50 and 500 U ml^{-1}) as described previously. After clarification 1 g of Eupergit-C beads (kindly donated by Rohm, Darmstadt, Germany) were added per 8 ml of enzyme solution. Holo-enzyme was incu-

bated with 2.4 mM TPP and 9 mM $MgCl_2$ to activate the enzyme prior to the addition of the Eupergit-C. After the addition of the beads a nitrogen head space was placed over the immobilisation reaction mixture. The mixture was shaken at 200 rpm for at least 24 h to allow the enzyme to bind to the support. Samples were assayed for residual transketolase activity.

After binding, any unbound enzyme was removed by washing the support three times with 50 ml of 10 mM phosphate buffer at pH 6.5 containing 2.4 mM TPP and 9 mM $MgCl_2$. Subsequently, the beads were recovered and any unreacted epoxide groups were capped with β-mercaptoethanol. The capping reaction was performed in 10 ml of 10 mM phosphate buffer at pH 6.5 containing TPP [2.4 mM], $MgCl_2$ [9 mM] and β-mercaptoethanol [10 mM] per 4 g (wet) beads. The capping reaction was allowed to proceed for 16 h under a head of nitrogen. After blocking with mercaptoethanol the beads were recovered and washed in 500 ml reverse osmosis water.

2.3.2. Amberlite XAD7

Amberlite XAD7 (Sigma–Aldrich) was treated with gluteraldehyde in order to provide the ligands to which protein can be covalently bound to the support according to the activation protocol developed by Carleysmith [19]. Beads were mixed for 16 h in a solution of 25% (w/v) gluteraldehyde at pH 12 (maintained by addition of 1 M NaOH). After activation with gluteraldehyde the beads were washed repeatedly in reverse osmosis water (500 ml) to remove any unreacted gluteraldehyde. In order to immobilise transketolase 1 g of beads were incubated for 24 h in the presence of clarified cell extract in 1 M phosphate buffer containing 200 U ml^{-1} transketolase. After immobilisation the beads were recovered by filtration and the supernatant assayed for residual transketolase activity. The recovered beads were washed in 15 ml aliquots of 10 mM phosphate buffer at pH 7.5 until no more transketolase was found in solution (normally 3 cycles).

2.4. Immobilised transketolase activity assay

The yield of immobilised enzyme activity was measured from the initial rate of L-erythrulose production during the first 20 min of a biotransformation with 100 mM reactants. Glycolaldehyde, β-hydroxypyruvic acid (lithium salt and free acid form) were obtained from Sigma–Aldrich while L-erythrulose hydrate (for standards) was supplied by Fluka Chemicals (Gillingham, Kent, UK). The reaction mixture contained glycolaldehyde [100 mM], hydroxypyruvic acid [100 mM], TPP [2.4 mM], $MgCl_2$ [9 mM] and 0.5% (w/v) mercaptoethanol (pH 7.5). One gram (wet) biocatalyst beads were added to 10 ml of the reaction mixture to initiate the reaction. The pH was maintained at 7.5 using 1 M HCl with a Radiometer Autotitrator. Samples (100 μl) from the biotransformation were diluted in 6 mM sulphuric acid to a suitable concentration, not exceeding 5 mM, and filtered to 0.2 μm prior to analysis.

2.5. Immobilised transketolase storage stability

The storage stability of immobilised transketolase was monitored by regular activity measurements after storage at 4°C in 100 mM phosphate buffer at pH 7.5 containing 1% (w/v) sodium benzoate to prevent microbial growth in the presence and absence of mercaptoethanol and a nitrogen headgas.

2.6. Immobilised transketolase operational stability

Several batches were performed sequentially, in a 10 ml stirred vessel. pH was maintained with a Radiometer Autotitrator as described previously. One gram (wet) biocatalyst beads were added to 10 ml 100 mM reaction mixture to initiate the reaction. The progress of the reaction was monitored by HPLC as described previously. The reaction carried out was in the presence and absence of 0.5% (w/v) mercaptoethanol as described in the text. Once the titrant

addition profile had reached a plateau the reaction was deemed to have reached completion. The reaction mixture was decanted off and the beads were washed twice with 10 ml of 100 mM phosphate buffer (pH 7.5). After washing, 10 ml of fresh reaction mixture was added and the reaction monitored by HPLC as described previously.

2.7. Stability of immobilised transketolase in the presence of glycolaldehyde

The stability of immobilised transketolase in the presence of glycolaldehyde was monitored and compared with the stability of the free enzyme as a control. The immobilised enzyme (10 g) was stored at 25°C in 50 ml phosphate buffer [10 mM] containing glycolaldehyde [500 mM], 0.5% mercaptoethanol (w/v), TPP [2.4 mM] and $MgCl_2$ [9 mM] at pH 7.5. Samples of beads (1 g) were removed and assayed for transketolase activity as described previously.

3. Results

3.1. Storage stability

In order to demonstrate whether transketolase was sufficiently stable in solution and therefore could be immobilised with a reasonable retention of activity, the storage stability of holo- and apo-transketolase was investigated. Transketolase samples containing 50 U ml^{-1} transketolase were prepared in the presence and absence of added cofactors. The results are shown in Fig. 2. Activated enzyme is rapidly denatured under immobilisation conditions. Although the apo-enzyme was stable for 72 h, the holo-enzyme lost all activity within a 24 h period. However, the activity of holo-transketolase could be maintained over a 72 h period by incubation of the enzyme and cofactor under a head of nitrogen or alternatively in the presence of 0.5% (w/v) mercaptoethanol. Both methods are known to stabilise proteins by preventing

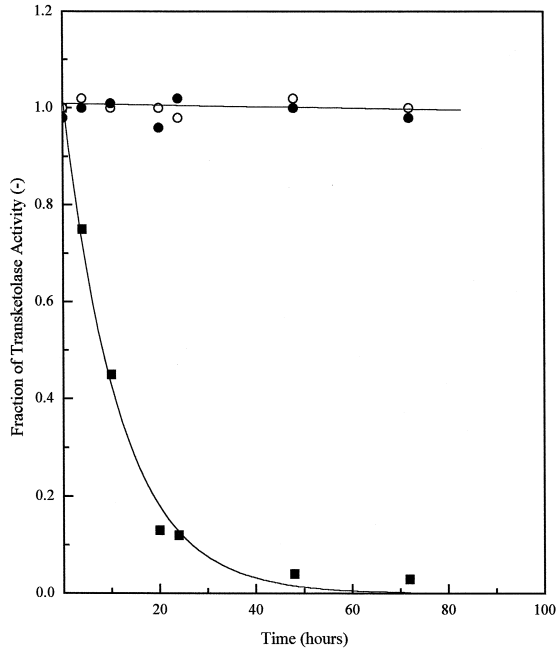

Fig. 2. Storage stability of free transketolase in its holo form (■), under a nitrogen headgas (○) and with 0.5% (w/v) mercaptoethanol in an oxygen rich environment (●).

oxidation of essential cysteine, methionine or tryptophan residues [15], indicating that oxidation of these residues is the main cause of inactivation of the soluble holo-enzyme.

In order to investigate the storage stability of immobilised transketolase, preparations of the immobilised enzyme were stored under a nitrogen head at 4°C. Activity measurements were made at regular intervals and indicated that the enzyme was stable under these conditions. In contrast to enzyme stored in the absence of nitrogen which rapidly denatured, the immobilised enzyme under a nitrogen head lost only 50% of its activity after 2 months storage.

3.2. Operational stability

3.2.1. Oxidation of holo-transketolase

Although the enzyme can be stored in its apo-form, synthetic uses of the catalyst will necessitate the presence of cofactors. In order to demonstrate whether immobilisation was able to stabilise the enzyme against oxidative denatura-

Table 1
Batch to batch stability of immobilised transketolase in the absence of mercaptoethanol as a stabilising agent during the biotransformation

Preparation	Batch 1		Batch 2		Batch 2/Batch 1
	Specific activity (U g^{-1})	Retained activity (%)	Specific activity (U g^{-1})	Retained activity (%)	(%)
XAD7	250	20	145	10	58
Eupergit	250	20	140	10	57

The table also gives a comparison between Eupergit-C® and XAD7 preparations (the Eupergit-C biocatalyst was prepared in the absence of TPP).

tion, two consecutive biotransformations were done in the absence of mercaptoethanol. All biotransformations were carried out with 100 mM reactants and in the presence of cofactors. The XAD7 and Eupergit-C® preparations both showed a loss of about 50% of their activity after the first batch operation (Table 1).

In order to demonstrate whether the presence of a reducing environment was able to enhance the operational stability of the immobilised enzyme, consecutive 100 mM biotransformations were done in the presence of 0.5% (w/v) mercaptoethanol (Fig. 3). No activity was lost during four consecutive biotransformations with the Eupergit-C immobilised biocatalyst. The rapid loss of activity of immobilised transketolase observed in the absence of added stabilisers demonstrates that the immobilisation of transketolase is unable to prevent the gradual oxidation of the holo-form of the enzyme.

3.2.2. Enzyme stabilisation against glycolaldehyde

If the biocatalyst is to be reused economically, its ability to withstand the denaturing effects of the toxic substrate (glycolaldehyde) is arguably one of its most important characteristics. To determine the effect of glycolaldehyde

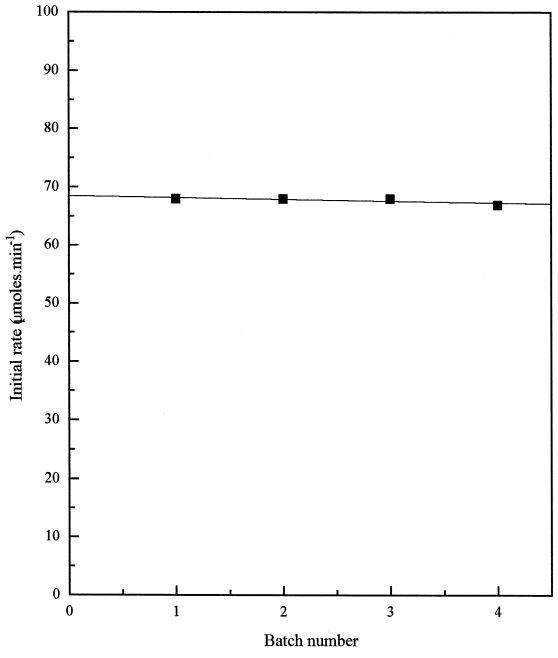

Fig. 3. Operational stability of Eupergit-C immobilised holo-transketolase in the presence of 0.5% (w/v) mercaptoethanol. Enzyme stability determined by activity measurement in sequential batches.

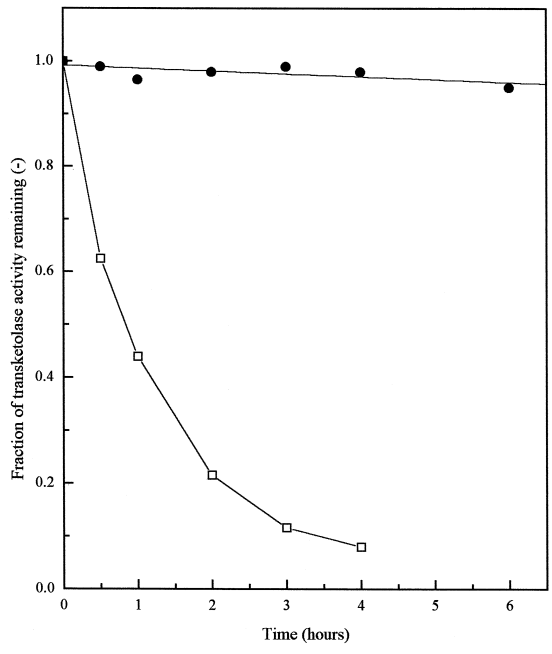

Fig. 4. Storage stability of Eupergit-C® immobilised (●) and free (□) holo-transketolase in the presence of 0.5 M glycolaldehyde.

Table 2
The long term stability of immobilised transketolase in 500 mM glycolaldehyde

Time (h)	Eupergit-C®		XAD7	
	Specific activity (U g^{-1})	Remaining activity (–)	Specific activity (U g^{-1})	Remaining activity (–)
0	1105	1	408	1
24	900	0.8	301	0.75
100	552	0.5	160	0.4
288	156	0.11	85	0.08

Comparing the remaining activities of XAD7 and Eupergit-C® preparations of the enzyme.

upon the stability of immobilised transketolase, beads were incubated in 500 mM glycolaldehyde under reaction conditions. Samples were taken for residual activity assay. The results are shown in Fig. 4 and indicate that no activity is lost by Eupergit-C® immobilised transketolase over 6 h of operation, while the free enzyme shows a significantly lower activity half life of less than 1 h under the same conditions.

Two successive biotransformations were done in the presence of 0.5% (w/v) mercaptoethanol with high concentrations of both hydroxypyruvic acid (500 mM) and glycolaldehyde (500 mM). There was no loss of enzyme activity between the two successive reactions in both preparations of immobilised transketolase.

In order to simulate some of the effects of operation, the immobilised enzyme was stored in glycolaldehyde under reaction conditions for an extended period of time. The results for the extended storage of immobilised transketolase are shown in Table 2. The table also compares the stability of XAD7 and Eupergit-C® preparations of transketolase. Both preparations demonstrated enhanced stability in the presence of glycolaldehyde. The half life of the preparations were determined from these data as being 80 and 100 h, respectively.

4. Discussion

Soluble transketolase, in both apo and holo forms, is susceptible to inactivation by glycolaldehyde and by the oxidation of essential amino acid residues involved in catalysis [14]. Likewise it has been proposed that inactivation by glycolaldehyde occurs as a result of Schiff's base formation between the aldehyde and amino acids on the surface of the enzyme [14]. Transketolase immmobilised onto both Eupergit-C and XAD7 showed increased stability in the presence of glycolaldehyde.

This stabilisation can be explained in two ways. First, the formation of Schiff's bases between glycolaldehyde and amino acid side chains on the surface of transketolase has been prevented or significantly reduced by the immobilisation of the enzyme. Alternatively, the formation of Schiff's bases with surface amino acids results in a change in the enzyme's three dimensional structure, which subsequently irreversibly inactivates the enzyme. This seems a likely explanation given that immobilising the enzyme prevents this structural reorganisation. Several researchers have demonstrated that immobilisation rigidifies the structure of proteins [4,5,18].

Regardless of the mechanism, the stabilisation is of significance to the operation of transketolase-catalysed conversions. The soluble enzyme must be operated in a reactor system at low concentrations of glycolaldehyde. This is achieved by feeding the substrate into the reactor at a rate such that the reactor concentration of aldehyde is kept at a minimum. However, this is no longer necessary in the case of the immobilised enzyme.

The inactivation of soluble transketolase by oxidation could not be prevented by immobilisation. Crystallographic studies of *E. coli* transketolase (1.9 Å resolution) indicate the presence

of cystine159 in the region of the TPP binding site containing a sulphoxide group [12] (Fig. 5). It is likely that the addition of mercaptoethanol to the medium or the use of a nitrogen head gas during immobilisation prevents oxidation of cysteine159.

Stabilisation of enzymes for synthetic potential can be achieved by a variety of methods including immobilisation, inclusion of stabilising solutes, chemical modification and protein engineering. An understanding of the mechanism of inactivation is necessary in order to determine the most effective strategy for stabilising the enzyme. Transketolase has been shown to be susceptible to denaturation by two effects, acting either independently or together: oxidation of essential amino acid residues and the toxic effects of glycolaldehyde. These data highlight the fact that a single method of stabilisation will not always result in a stable biocatalyst. The reactor environment is complex, containing several components each of which can

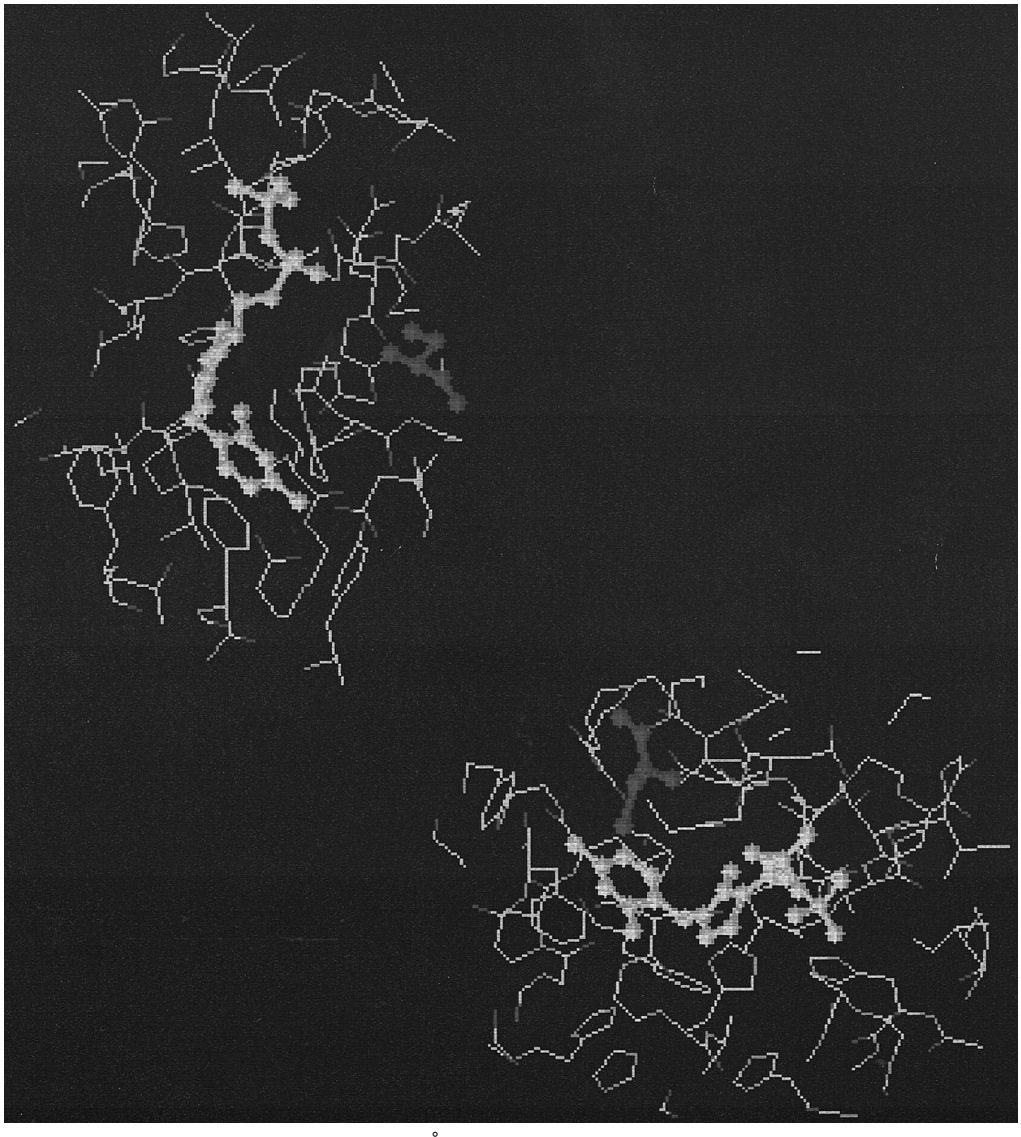

Fig. 5. Wireframe diagram of the region within 8.0 Å of TPP, cystine159 has been dark shaded and TPP light shaded [12].

reduce the stability of the enzyme. Therefore, it follows that several inactivation methods can be acting upon the enzyme simultaneously. As a result each inactivation method should be isolated and a strategy for stabilising the enzyme against each inactivation mechanism should be developed.

5. Conclusions

Immobilisation onto both XAD7 and Eupergit-C® was able to stabilise transketolase against inactivation by its substrate glycolaldehyde. While immobilisation (onto either support) was not able to prevent the gradual inactivation of the holo-enzyme by oxidation, the inclusion of a stabilising solute (mercaptoethanol) was able to significantly reduce the oxidative inactivation. The use of the two stabilising methods together could be used to successfully stabilise the enzyme in the reactor.

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